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ESTIMATION OF ANTIBACTERIAL AND ANTIOXIDANT ACTIVITIES OF PHYCOCYANIN ISOLATED FROM *Spirulina*

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ABSTRACT: The objective of the current research was to explore chemical characterization, antibacterial and antioxidant activity of phycocyanin isolated from *Spirulina.* Two bands correspond to low molecular weights between 15 and 25 kD were isolated using SDS-PAGE. These two low molecular weight (>15 kD and <25 kD) bands probably represent the pure phycocyanins. The contents of the hydrophobic amino acid residues (Pro, Gly, Ala, Val, Ile, Leu, Phe) were around 41.28% (16.02+ 3.08+ 2.84+ 3.14+ 3.2+ 9+ 4, respectively) of the total amino acids. The minimum inhibitory concentration (MIC) of phycocyanin against the three studied bacteria (*Staphylococcus aureus*, *Aeromonas hydrophila* and *Salmonella enteritidis*) was 10 µg/ml. The studied material did not have any effect against *Enterococcus faecalis* at the same concentration (0-320 µg/ml). The respective scavenges capacity of samples (SC_{50}) values were determined and the SC_{50} value of the phycocyanin was 104 µg/ml. According to the results it could be concluded that phycocyanin was may be used in manufacturing of new functional products with increased nutritional value as the first step toward increasing its consumption and preserving food systems, as it is rich in nutrients and lower in cost.

Key words: *Spirulina*, phycocyanin, antibacterial, antioxidant, cyanobacteria.

INTRODUCTION

In recent years the consumer concern in relation to health and safety issues about the use of synthetic colorants in foods has increased. The Food and Drug Administration (FDA) in USA, the European Food Safety Authority (EFSA) in Europe, and many other national authorities around the world have restricted the use of synthetic colorants in foods, confectionery and beverages because of their confirmed or suspected association with increased cancer development or induction of allergic reactions. For the same reasons other colorants are under study and are only provisionally allowed. The tendency in food manufacturers is therefore going progressively toward the use of natural additives. Among the different colors, the confectionary and drinks industry has a high demand in blue colorants, however, they are uncommon in nature thus leading to the use of synthetic ones. For this reason the food industry is now expressing a growing interest in the search, use, and stabilization of natural blue colorants. The cyanobacterium (*Spirulina*), known mainly as a source of nutraceuticals, has recently gained considerable attention also as a source of blue pigment **(Belay *et al*., 1993)**. Phycocyanin is an accessory photosynthetic pigment of the phycobiliprotein family. In general, phycobiliproteins are made up of chromophore-bearing polypeptides containing α and β subunits, which have a molecular weight of around 20 kDa (**Abalde *et al*., 1998**). Phycobilisome from the cyanobacterium *Spirulina* sp. consists of allophycocyanin (APC) cores surrounded by c-phycocyanin (CPC) peripherally. CPC is the major phycobiliprotein in *Spirulina* and constitutes up to 20% of its dry weight (**Vonshak, 1997; Jaouen *et al*., 1999**). Phycocyanin is a natural blue colorant, and has been used as a colorant in health drinks, beverages, confectionary and cosmetics. Small

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quantities are also used as biochemical tracers in immunoassays due to its fluorescent properties **(Silveira *et al*., 2007)**. Nowadays, there has been an increasing awareness on cyanobacteria as a possible source for new drugs. Recent studies on cyanobacterial bioactive compounds showed antibacterial, antiviral, antimalarial, anticancer, antimicrobial, antifungal or anti-inflammatory, immunosuppressive and pharmacological activities **(Romay *et al.*, 2003; Mayer and Hamann, 2005; Eriksen, 2008; Sitohy *et al*., 2015)**. In fact, for the last 50 years, high levels of antibiotics are commonly used for treatment and prevention of infectious diseases in humans and animals. This led to emergence and dissemination of resistant bacteria and resistance genes in wild populations posing a world-wide problem in human and veterinary medicine and compelling to develop antibiotics **(Van den Bogaard and Stobberingh, 2000)**. Antibacterial phycocyanin from *Anabaena oryzae* SOS13 has been reported **(Sitohy *et al*., 2015)**. Development of suitable antioxidant molecule is gaining more importance in present days as it plays a key role in preventing or delaying hepatotoxicity, heart diseases and cancer. Butylhydroxyanisole (BHA) and butylhydroxytoluene (BHT) are the generally used artificial antioxidants and possess potential health risks and toxicity. The use of artificial oxidants has reduced due to their carcinogenic nature. So, there is crucial need to substitute them with new harmless natural antioxidants. Antioxidants can also be used industrially in food and cosmetics as preservatives.

As a consequence, this work was conducted to evaluate the antimicrobial activity of phycocyanin extracted from *Spirulina* using agar well-diffusion assay and minimum inhibition concentration (MIC) against selected Gram positive and Gram negative bacteria. The antioxidant activity of Spirulina phycocyanin was further explored.

MATERIALS AND METHODS

Micro-organism and culture preparation

The cyanobacterial isolate (*Spirulina)* used in the present study was previously obtained from Dr. Ali Salama Assistant Professor of Microbiology, Microbiology Department, Faculty of Agriculture, Zagazig University, Egypt. The fresh *Spirulina* culture was prepared by inoculating 250 ml of $BG^{0}11$ medium with 10 ml of 10 days old culture in 500 ml Erlenmeyer flasks. Inoculated flasks were incubated at 26 ± 2˚C for 30 days under continuous illumination (600-800 lux) using 36W white fluorescent lamp.

Phycocyanin Extraction Purification

Biomass preparation and extraction

Phycocyanin was extracted from the fresh *Spirulina* strain biomass using the modified methods of **Sarada *et al.* (1999)**. Thirty day grown *Spirulina* cells were harvested by centrifugation at 3000 *x*g for 5 min (Jouan, MR 1822, France) at 20 °C. Cell pellets were washed with 1M Tris–HCl buffer (pH 8.1). One volume of washed cell mass was re-suspended in five volumes of the same buffer and treated for the extraction of phycocyanin using physically method according to **Sitohy *et al.* (2015)** (freezing at -50˚C and thawing at 25˚C). The content was then centrifuged at 5000 *xg* for 10 minutes and then the supernatant was collected and stored in the refrigerator.

Purification procedure of C-PC was followed as described by **Soni *et al.* (2006)**. Finely powdered ammonium sulfate was gradually added into the crude extract of phycocyanin to obtain 20% saturation and kept under continuous stirring for 1 hr. The resulting solution was kept overnight and centrifuged at 17,000 *xg* for 20 min. The supernatant was pooled and subjected to 70% ammonium sulfate saturation as previously described by **Sitohy *et al.* (2015)**. After overnight incubation, the solution was centrifuged at 17,000 *xg* for 20 min and the resulting pellets were resuspended in a small quantity of 20 mMTris–Cl buffer (pH 8.1) and subjected to dialysis for 48 hr., against 100 times volume of the same buffer, with a change of buffer four times.

The levels of the phycocyanins (phycocyanin (C-PC), allophycocyanin (C-APC) and phycoerythrin (C-PE) concentrations were determined using the spectrophotometric method of **Boussiba and Richmond (1979)**. The contents of phycocyanin components were calculated according to the following equations:

$$C-PC\left(\frac{mg}{ml}\right)=\frac{OD620-0.7OD650}{7}.38 \quad (A)$$

$$C-APC\left(\frac{mg}{ml}\right)=\frac{OD650-0.19OD620}{5}.65 \quad (B)$$

$$C-PE(mg/ml)=(OD540-2.8[C-PC]-1.34[C-APC])/12.7(C)$$

Chemical Characterization of Phycocyanin

SDS-PAGE

SDS–PAGE was performed on a discontinuous buffered system according to **Laemmli (1970)**. Stacking and separation gels (3% and 12%) were prepared from 30% acrylamide and 0.8% N, N-bis methylene acrylamide solution. The electrode buffer (pH 8.3) contained 0.025 mol Tris, 0.192 mol glycine, and 0.1% SDS. Five milligrams of phycocyanin were dispersed in 1 ml of 0.03 M Tris buffer (pH 8.0) for 15 min with vortexing and the extract was then centrifuged for 10 min at 11000 x *g*. An aliquot of the extract (20 µl) was mixed with 20 µL of SDS-sample buffer, heated at 96 °C for 3 min and an aliquot (10 µl) from the final mixture was electrophoresed. After running at 10 and 20 mA (at the stacking gel and running gels, respectively), staining was performed with Coomassie Brilliant Blue R-250 dye. The molecular weight of bands was calculated using the corresponding protein marker (10-500 KD).

Quantitative determination of total amino acids

Total amino acids composition of phycocyanin was determined by amino acid analyzer apparatus model "Eppendorf LC3000" using the following steps: A known weight (0.2 g) of phycocyanin was added to 10 ml 6 N hydrochloric acid in a sealing tube, and then placed in oven at 110°C for 24 hours. Hydrolysates were transferred quantitatively into a porcelain dish and the hydrochloric acid was then evaporated to dryness at 50-60°C on a water bath. Distilled water (5 ml) was added to the hydrolysate and then evaporated to dryness to remove the excess of hydrochloric acid and finally the residue was dissolved in 10 ml distilled water and filtrate through 0.45 mm filter. The filtrate was dried under vacuum with a rotary evaporator, then 10 ml of distilled water were added and the samples were dried a second time. One ml of 0.2 N sodium citrate buffer at pH 2.2 was added and the samples were stored frozen in a sealed vial until separation of amino acids by amino acid analyzer (Column: hydrolysate column Eppendorf LC 3000 (250 x 4.6). The amino acid analyzer condition were; temperature was 47°C; Sample: 20 µl; Buffer system: Sodium acetate, buffer A (pH 3.3), buffer B (pH 3.6), buffer C (pH 4.3) and buffer D (pH11.0); Flow rate: 0.2 ml/min.). Ninhydrin was used for the detection of amino acids at 440 nm for proline and 570 nm for the other amino acids through an oxidative decarboxylation reaction. The peak area and percentage of each amino acid were calculated by computer software AXXIOM CHROMATOGRAPHY-727.

Antibacterial activity of phycocyanin

The antibacterial activity of phycocyanin isolated from *spirulina* was tested against Gram positive (*Staphylococcus aureus* and *Enterococcus faecalis*) and Gram negative bacteria (*Aeromonas hydrophila* and *Salmonella enteritidis*) using agar well diffusion method and turbidity liquid media assay.

Minimum inhibitory concentration

Minimum inhibitory concentration (MIC) was determined using agar well diffusion method (**Nanda and Saravanan, 2009**). The pure cultures of bacterial strains were sub-cultured on nutrient broth at 37˚C on a rotary shaker at 200 rpm. The exponential phase cultures of these strains were adjusted to a concentration of 1×10^9 CFU ml^{-1}. Each strain was spread uniformly onto the individual plates using sterile cotton swabs. Wells of 6-mm diameter were made on Nutrient agar plates using a gel puncturing tool. Aliquots (30 µl) of phycocyanin (0, 5, 10, 20, 40, 80, 160 and 320 µg/ml) were transferred into each well. After incubation at 37 ˚C for 24 hr., the diameter of the inhibition zones were measured using a transparent ruler (**Ehinmidu, 2003**). MIC of an antimicrobial is taken as the lowest concentration (µg/ml) that will inhibit the visible growth of a microorganism after overnight incubation.

Bacterial growth curve (turbidity test)

Turbidity (A_{600}) was used to estimate CFU/ml in nutrient broth media suspensions as

an indicator of the bacterial growth over time. Phycocyanin (at their MIC= 10 µg/ml) was added to the medium (10 ml) containing 100 µl G+ or G− bacteria (10^9CFU/mL) and examined for their growth as compared to control (without adding any substance). All treatments were incubated at 37°C for different time periods (0, 6, 12, 18 and 24 hr.) before measuring the turbidity

Antioxidant Activity Evaluation (Dpph Radical-Scavenging Activity)

The antioxidant activity of phycocyanin at different concentrations (40, 80, 160, 320, 640, 1250 and 2500 µg/ml) isolated from *spirulina* was evaluated by their ability to scavenge DPPH radicals according to the method described by **Hatano *et al.* (1988)**. Five hundred µL of each concentration were added to 3 mL of 0.1 mM DPPH dissolved in ethanol. After incubation period of 30, min at room temperature, the absorbance was determined against control at 517 nm.

The radical scavenging capacity of the samples was measured as a decrease in the absorbance of DPPH radical and was calculated using the following equation.

$$\text{Radical scavenging activity (\%)} = \frac{\text{Abs. control} - \text{Abs. sample}}{\text{Abs. control}} \text{x100}$$

The SC_{50} (the concentration of the sample that scavenges 50% of the DPPH radicals) was calculated by linear regression of curves showing percentage scavenging versus sample concentration.

RESULTS AND DISCUSSION

Chemical Characterization of Phycocyanin

Cyanobacterial phycobiliproteins are classified into three main groups: phycocyanin (C-PC), phycoerythrin (C-PE) and allophycocyanin (C-APC) depending on inherent colour and absorbance properties. Here prefix ‘‘C’’ to the abbreviation of each phycobiliprotein indicates its cyanobacterial origin. The absorption maximam for C-PC, C-PE and C-APC are between 610- 620, 540 - 570, and 650 - 655 nm, respectively (**Reis *et al.*, 1998; Bermejo *et al.*, 2003**). The levels of the phycocyanins (C-PC, C-APC and C-PE) concentrations were observed in Table 1. The obtained result showed its highest concentrations of the three phcocynin components in the following order; C-PC > C-APC > C-PE (.0.098 ±0.009, 0.064 ±0.008, 0.014 ±0.006 mg/ml, respectively). The results obtained by **Sitohy *et al.* (2015)** were 112.7, 70.9 and 16.9 µg/ml for C-PC > C-APC > C-PE, respectively.

SDS-PAGE gel photo of phycocyanin isolated from *Spirulina* was observed in Fig. 1. Two bands correspond to low molecular weights were detected between 15 and 25 kD. These two low molecular weight (>15 kD and <25 kD) bands probably represent the pure phycocyanin which are in accordance with the results of **Santiago-Santos *et al.* (2004)** who defined the molecular weight of the two comparable SDS-PAGE bands as 17 and 21 kD each, while the estimated molecular mass of native purified phycocyanin was 114 kD. These results are consistent with **Sitohy *et al.* (2015)**.

The amino acids composition of phycocyanin isolated from *Spirulina* were listed in Table 2. The contents of the hydrophobic amino acid residues (Pro, Gly, Ala, Val, Ile, Leu, Phe) is around 41.28 % (16.02+ 3.08+ 2.84+ 3.14+ 3.2+ 9+ 4, respectively) of the total amino acids. The content of the acidic amino acids residues (asp + glu; 18.45 + 13.46, respectively) is higher than that of the basic amino acids (arg + lys + his; 7.8 + 5.4 + 1.5, respectively). In Gram-negative bacteria this adsorption is followed by insertion of the peptides into the outer membrane structure stimulated by hydrophobic interaction (**Sitohy *et al.*, 2015**) the antibacterial peptides then cause destruction and permeabilisation of cytoplasmic membrane.

Antibacterial activity of phycocyanin

The minimum inhibitory concentration (MIC) of phycocyanin isolated from *Spirulina* was determined against Gram+ (*Staphylococcus aureus* and *Enterococcus faecalis*) and Gram- (*Aeromonas hydrophila* and *Salmonella enteritidis*) bacteria using agar well diffusion assay under the influence of different concentrations (0-320 µg/ml) (Table 3). The results indicated concentration dependent inhibition zone diameters for tested materials.

Table 1. Extraction yield of Phycocyanins (mg/ml) from *Spirulina* using combination of freeze-thaw treatments (-50 °C × 25 °C).

Phycocyanin components	Concentration (µg /ml)
C-PC	98 ±9
C-APC	64 ±8
C-PE	14 ±6

C-APC: allophycocyanin; C-PC: c-phycocyanin; C-PE: phycoerythrins

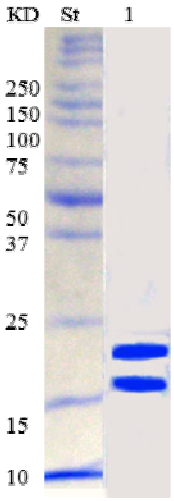

Fig. 1. SDS-PAGE of phycocyanin isolated from *Spirulina* (lane 1)

Table 2. Amino acid composition (g amino acid/100 g phycocyanin protein) isolated from *Spirulina*

Amino acid	Concentration (g / 100 g phycocyanin)
Aspartic	18.45
Threonine	3.26
Serine	6.55
Glutamic	13.46
Proline	16.02
Glycine	3.08
Alanine	2.84
Cystine	0.52
Valine	3.14
Methionine	---
Isoleucine	3.2
Leucine	9
Tyrosine	0.65
Phenylalanine	4
Histidine	1.5
Lysine	5.4
Arginine	7.8

Table 3. Inhibition zone diameter (mm) of phycocyanin at different concentrations against Gram positive and Gram negative bacteria

Microorganisms	Inhibition zone diameter (mm)							
	Phycocyanin concentration (µg/ml)							
	0	5	10	20	40	80	160	320
	Gram positive							
Staphylococcus aureus	0 ±0	0 ±0	19 ±0.8	21 ±0.9	25 ±1.1	26 ±1.3	30 ±2	31 ±1.5
Enterococcus faecalis	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0
	Gram negative							
Aeromonas hydrophila	0 ±0	0 ±0	17 ±0.5	20 ±0.6	21 ±0.7	22 ±0.7	23 ±0.9	30 ±1.2
Salmonella enteritidis	0 ±0	0 ±0	19 ±0.8	20 ±0.4	21 ±0.7	26 ±0.8	28 ±1	29 ±1.1

The minimum inhibitory concentration (MIC) of phycocyanin against the three studied bacteria (*Staphylococcus aureus*, *Aeromonas hydrophila* and *Salmonella enteritidis*) was 10 µg/ml.

The studied material did not have any effect against *Enterococcus faecalis* at the same concentrations (0-320 µg / ml)

Four tested G+ (*Staphylococcus aureus*) and G- (*Aeromonas hydrophila* and *Salmonella enteritidis*) bacteria were grown in their appropriate liquid media in the presence or absence of the MIC of phycocyanin (10 µg/ml) for 24 h at 37°C (Fig. 2).

The growth curves of the tested control bacteria reached a maximum turbidity after around 24 hr., at 37°C. At this time point, phycocyanin reduced the growth of *Staphylococcus aureus, Aeromonas hydrophila and Salmonella enteritidis* bacteria by about 74.54, 71.4 and 63.79 %, respectively.

The antimicrobial action of proteins phycocyanin may be initiated by an electrostatic interaction between their positively charged regions and the negatively charged regions of cell wall or cell membrane accompanied by a hydrophobic interaction between alike regions of the two reactants. Oscillating random Brownian motion of protein macromolecules attached to the cell walls and membranes may cause their stretching, producing big-sized pores, pore channels and cell wall and cell membrane disintegration, engendering higher cell permeability leading finally to cell emptiness, lysis and death (**Lavalette *et al*., 1999**). Based on the results of amino acid analysis reflect the hydrophobic nature of the phycocyanin and high activity as an antibacterial agent.

This phenomenon was in agreement with that found by **Ozdemir *et al*. (2004) and Muthulakshmi *et al*. (2012)** showing that *Spirulina* phycocyanin exhibited great potential antibacterial activities against five bacterial strains, but with less inhibition zones in the range of 7–13 mm.

Antioxidant Activity of Phycocyanin

DPPH radical scavenging activity (RSA) of phycocyanin at different concentrations (40, 80, 160, 320, 640, 125 and 2500 µg/ml) was observed in Fig. 3. The respective SC_{50} values were determined and the calculated SC_{50} value of the phycocyanin was 104 µg/ml.

The DPPH radical scavenging activity assay is one of the *in vitro* methods for the measurement of the capacity of an antioxidant to reduce free radicals. The degree of color changes is correlated with the sample antioxidant activity (**Xie *et al.*, 2008**).

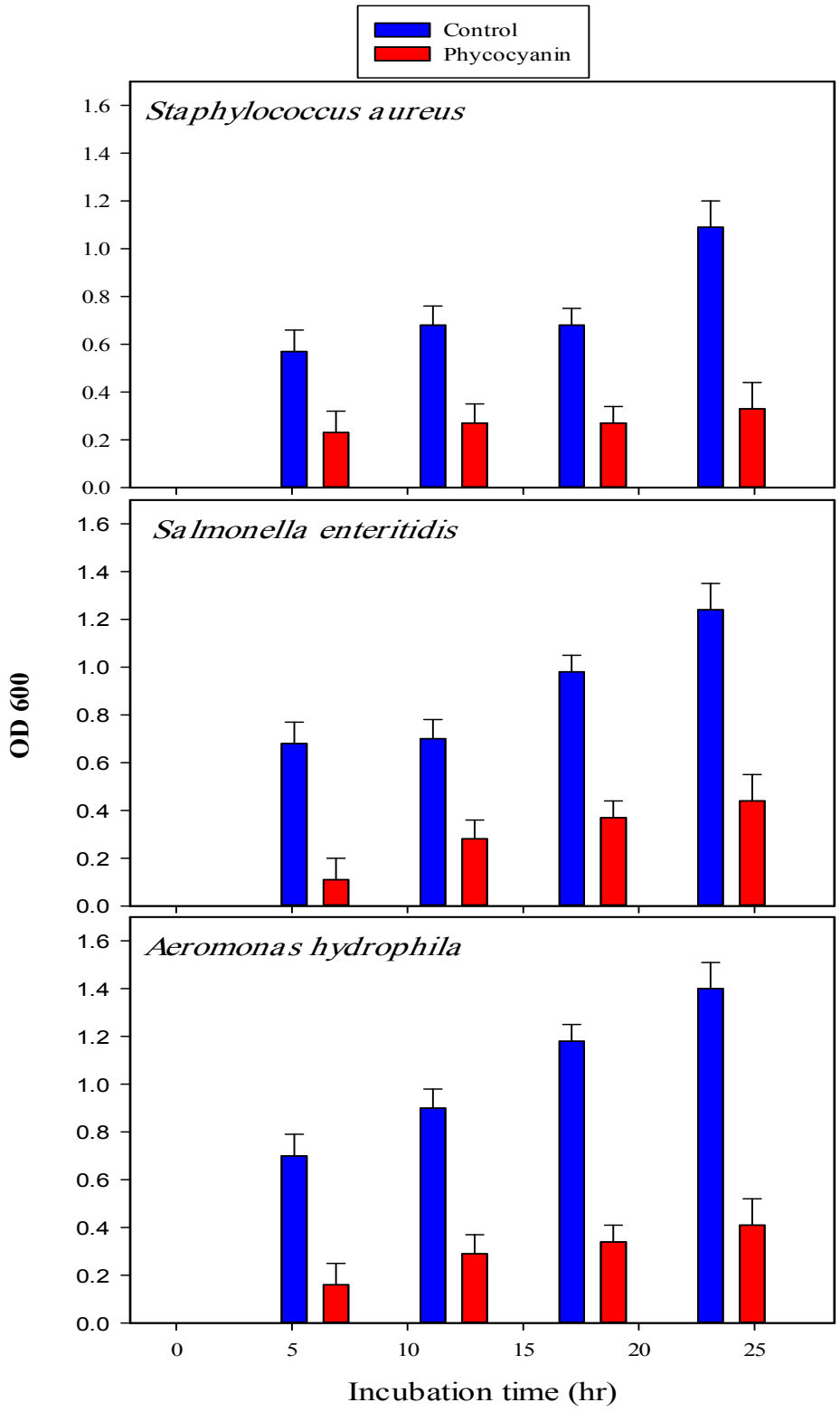

Fig. 2. Growth curve of G+(*Staphylococcus aureus*) and G-(*Slamonella enteritidis* and *Aeromonas hydrophila*) bacteria during 24 hr., at 37°C in the presence of 1 MIC of phycocyanin (10 µg/ml)

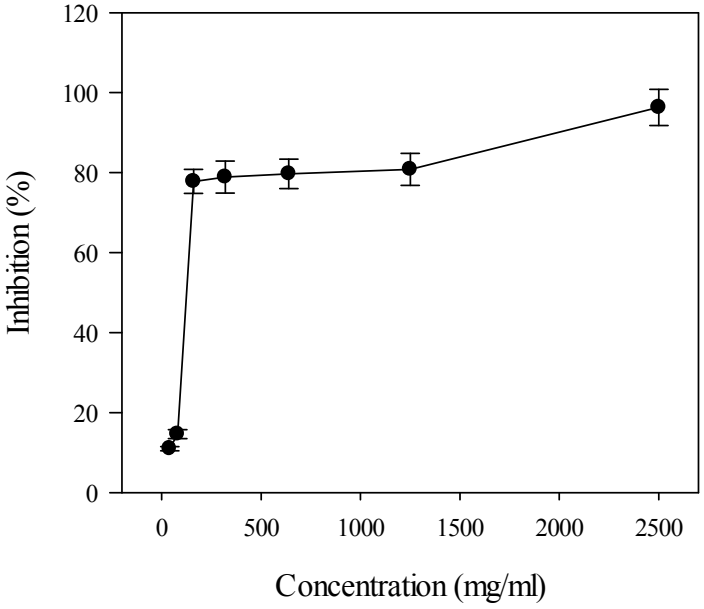

Fig. 3. DPPH radical scavenging activity (RSA) of phycocyanin at different concentrations (40, 80, 160, 320, 640, 125 and 2500 µg/ ml)

Some amino acids, such as histidine, tyrosine, methionine, and cysteine, have been reported to show antioxidant activity. In particular, histidine exhibited strong radical scavenging activity due to the rearrangement of its imidazole rang (**Xie *et al*., 2008**). **Sarmadi and Ismail (2010)** described the mechanisms of action of amino acids: and they showed that aromatic amino acids convert radicals to stable molecules by donating electrons while keeping their own stability: Also the hydrophobic amino acids enhance the solubility of peptides in lipids, which facilitates accessibility to hydrophobic radical species; while the acidic and basic amino acids contain carboxyl and amino groups in their side chains, which act as chelators of metal ions and as hydrogen donors. Dietary use of antioxidants has been shown to promote health by increasing antioxidant capacity (**Samaranayaka and Li-Chan, 2011**).

According to the results it could be concluded that phycocyanin was may be used in manufacturing of new functional products with increased nutritional value as the first step toward increasing its consumption and preserving food systems, as it is rich in nutrients and lower in cost.

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تقديـــر نشاط الفيكوسيانين المعزول من سبيرولينا كمضاد للبكتيريا ومضاد للأكسدة

شاهنده عبدالعزيز محمد - على عثمان - أحمد محمد أبوعيطه - محمود زكى سطوحى

قسم الكيمياء الحيوية الزراعية - كلية الزراعة - جامعة الزقازيق - مصر

الهدف من هذا البحث هو دراسة التوصيف الكيميائي، والنشاط المضاد للبكتيريا والمضاد للأكسدة للفيكوسيانين المعزول من طحلب سبيرولينا، ظهر فى التفريد الكهربى للبروتين أثنين من تحت الوحدات ذات أوزان جزيئية منخفضة تتراوح مابين ١٥ و ٢٥ كيلودالتون وهما يمثلان الفيكوسيانين النقى، أظهرت نتائج تحليل الأحماض الأمينية وجود ٤١,٢٨% من الأحماض الأمينية الهيدروفوبية وهى لها أثر كبير فى النشاط المضاد للبكتيريا حيث كان الحد الأدنى للتركيز المثبط ١٠ ميكروجرام/ملل، كما أظهرت نتائج مضادات الأكسدة أن التركيز الذى يثبط ٥٠% من الشقوق الحرة هو ١٠٤ ميكروجرام/ ملل، نستنتج من الدراسه ان الفيكوسيانين ممكن أن يدخل فى تصنيع الأغذية الوظيفية ذات القيمة الغذائية العالية كملون ازرق طبيعى كخطوة مبدئية تمهيدا لاستهلاكها حيث أنه يمكن أن يستخدم فى إطالة مدة حفظ الغذاء نظرا لدوره كمضاد للأكسده.

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